RESEARCH ARTICLE

Interannual variation in connectivity and comparison of effective population size between two splittail (*Pogonichthys macrolepidotus*) populations in the San Francisco Estuary

Brian Mahardja · Bernie May · Frederick Feyrer ·
Robert Coalter · Nann Fangue · Ted Foin ·
Melinda R. Baerwald

Abstract The discovery of two genetically distinct splittail populations within the San Francisco Estuary, one which spawns in the rivers of the Central Valley and another in the Petaluma and Napa Rivers of the San Pablo Bay, prompted the need to evaluate their degree of connectivity and relative sizes. We genotyped multiple age-0 splittail cohorts using 19 microsatellite loci to assess any spatiotemporal changes in the distribution of the two populations and estimate their effective population sizes (N_e). Genetic population assignments demonstrated that while age-0 splittail are predominantly spatially segregated by populations, substantial geographical overlap may occur during years of high precipitation. However, despite this periodic range overlap, the original observed population structure has persisted for nearly a decade which has included a similarly wet year. This suggests that the present population structure will likely persist in the future due to strong philopatry and/or adaptive differences. We also found that N_e estimates were generally lower for the San Pablo Bay population than the Central Valley population, which is consistent with the relative amount of habitat availability in the two locations and genetic diversity indices. The relative isolation and apparent lower N_e of the San Pablo Bay splittail population indicates a higher vulnerability to extinction. A more consistent monitoring effort of splittail in the Petaluma and Napa Rivers may be necessary in order to better understand the future viability of this less studied population.

B. Mahardja (✉) · B. May · M. R. Baerwald
Genomic Variation Laboratory, Department of Animal Science, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA
e-mail: bmahardja@ucdavis.edu; brian.mahardja@water.ca.gov

B. May
e-mail: bpmay@ucdavis.edu

M. R. Baerwald
e-mail: mrbaerwald@ucdavis.edu

Present Address:
B. Mahardja
Aquatic Ecology Section, California Department of Water Resources, 3500 Industrial Boulevard, West Sacramento, CA 95691, USA

F. Feyrer
United States Bureau of Reclamation, Bay Delta Office, 801 I Street, Suite 140, Sacramento, CA 95814, USA
e-mail: ffeyrer@usgs.gov

Present Address:
F. Feyrer
California Water Science Center, United States Geological Survey, 6000 J Street, Placer Hall, Sacramento, CA 95819-6129, USA

R. Coalter · N. Fangue
Department of Wildlife, Fish, and Conservation Biology, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA
e-mail: bcoalterjr@gmail.com

N. Fangue
e-mail: nafangue@ucdavis.edu

T. Foin
Department of Plant Sciences, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA
e-mail: tcfoin@ucdavis.edu

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Introduction

Variation in environmental attributes has been known to exert strong influence on a species' population dynamics. This pattern is especially common within estuaries, as water conditions (e.g. salinity, temperature, and turbidity) fluctuate spatiotemporally due to interactions between ocean tidal inflow and river freshwater outflow. The biota of the San Francisco Estuary possesses one of the strongest and most consistent relationships between abundance and freshwater outflow among large estuaries (Kimmerer 2002, 2004). Among these is the splittail (*Pogonichthys macrolepidotus*), an endemic San Francisco Estuary cyprinid fish species (Moyle 2002) known to exhibit a strong response to annual freshwater outflow (Sommer et al. 1997; Moyle et al. 2004). The splittail represents the sole surviving member of its genus owing to the extinction of the Clear Lake splittail (*Pogonichthys ciscoides*) in the early 1970s (Moyle 2002), and has been a subject of research interest as a federal and state species of special concern (Sommer et al. 2007).

Similar to other fishes native to the western United States (e.g., Colorado pikeminnow *Ptychocheilus lucius*, hitch *Lavinia exilicauda*), splittail exhibit migratory behavior (Moyle 2002). Adult splittail generally reside in brackish habitats of the upper San Francisco Estuary but migrate upstream to inundated freshwater floodplains and river margins to spawn during the wetter parts of the year (Daniels and Moyle 1983; Sommer et al. 1997; Moyle et al. 2004). Spawning mainly occurs in floodplains, but may also take place among river margins and backwater habitats at the cost of reduced growth rate (Feyrer et al. 2007). Larvae and juveniles typically reside in these upstream locations until water levels begin to recede in late spring, which promotes a downstream migration towards the brackish portions of the estuary (Feyrer et al. 2005). Splittail will generally remain in brackish water until sexual maturity is reached at about 2 years and subsequently initiate their first spawning migrations (Daniels and Moyle 1983; Moyle 2002).

No stock-recruitment relationship is found for splittail, likely due to the species' life history strategy and the high habitat variability of the San Francisco Estuary. Rather than being dependent on the success of previous cohorts, splittail year-class strength is positively correlated with freshwater outflow during the spawning season, where wet conditions coincide with high recruitment and dry conditions coincide with low recruitment (Sommer et al. 1997; Moyle et al. 2004). However, the role of freshwater flow in splittail persistence may be more complex than previously thought with the discovery of two genetically distinct populations: one spawning in the Central Valley (covering Sacramento River and San Joaquin River drainages and hereafter referred to as the Central Valley population), and another spawning in Petaluma and Napa Rivers (hereafter referred to as the San Pablo Bay population) (Baerwald et al. 2007). While freshwater habitat is available year-round for the Central Valley population, Petaluma and Napa Rivers remain mainly brackish throughout parts the year. A recent study suggests that some age-0 San Pablo Bay splittail may possess the ability to rear in brackish water (Feyrer et al. 2010), despite previous evidence of juvenile splittail's relatively high sensitivity to salinity (Young and Cech 1996). With the 2010 listing of the two splittail populations as distinct population segments (DPS) by the United States Fish and Wildlife Service (USFWS), it has then become essential to better understand the dynamics between the two genetically isolated populations (Baerwald et al. 2008; USFWS 2010).

The goal of our study is two-fold: (1) to investigate spatiotemporal changes in the distribution of the two splittail populations, and (2) to determine their respective genetic effective population size (N_e). N_e is an important parameter for informing the genetic health of populations, and its estimation provides an alternative to rigorous mark-recapture study for early detection of population fragmentation (England et al. 2010) and population decline (Antao et al. 2011). To monitor for changes in splittail population structure, population assignment analysis was conducted on age-0 fish collected from various time points between 2002 and 2012 using 19 microsatellite markers. N_e, defined as the size of an ideal population that experiences the same rate of genetic drift as the observed population (Wright 1931; Charlesworth 2009), and N_b (effective number of breeders), defined as the size of an ideal population exhibiting the same rate of genetic drift as the observed breeding adult cohort, were assessed for each population using temporal and linkage disequilibrium estimators.

Methods

Sample collection

Tissue samples (caudal fin clips) were taken from age-0 splittail collected in Petaluma and Napa Rivers by beach seine sampling between June and August of 2011 and 2012 (Fig. 1; Table 1). Collection attempts at these locations were successful with the exception of the Petaluma River during 2012, where we failed to capture any splittail. Age-0 splittail fin clips were also obtained from China Camp State
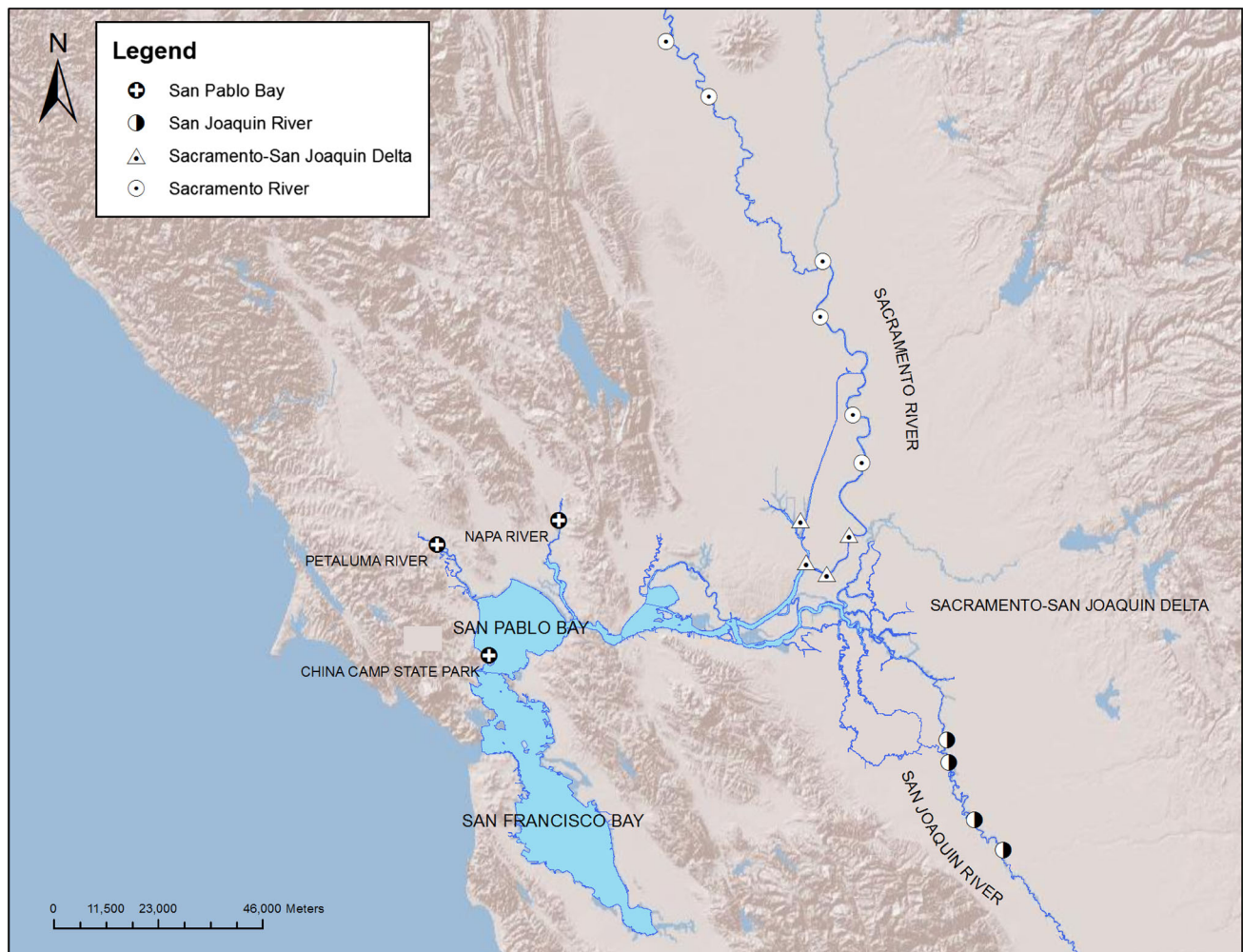
Fig. 1 Map overview of 2011–2012 sampling locations partitioned by region

Table 1 Sample size (N) sorted by location and collection year with their respective population assignment analysis results: San Pablo Bay (SPB), Central Valley (CV), and admixed (AD)

Region	2002				2003				2011				2012			
	N	SPB	CV	AD	N	SPB	CV	AD	N	SPB	CV	AD	N	SPB	CV	AD
San Pablo Bay																
Petaluma River	43	42	0	1	84	80	0	4	293	110	162	21	–	–	–	–
Napa River	33	32	0	1	52	36	14	2	89	22	65	2	404	388	2	14
China Camp State Park	–	–	–	–	–	–	–	–	3	0	3	0	–	–	–	–
Central Valley																
Sacramento-San Joaquin Delta	–	–	–	–	–	–	–	–	88	0	85	3	–	–	–	–
Sacramento River	59	1	53	5	79	0	79	0	89	0	89	0	–	–	–	–
San Joaquin River	44	0	40	4	32	0	30	2	135	1	128	6	–	–	–	–

Park and various locations throughout the Central Valley between June and July of 2011 by USFWS as part of their annual Delta Juvenile Fish Monitoring Program. Samples collected from the Central Valley were pooled into three regions for subsequent analysis due to the large number of sites: the Sacramento-San Joaquin Delta region, Sacramento River, and San Joaquin River. Age-0 splittail obtained in similar fashion and from similar locations

between May and June of 2002 and 2003 were also included. Full description of the collection method for 2002 and 2003 specimens can be found in Feyrer et al. (2005) and Baerwald et al. (2007).

Microsatellite genotyping

DNA was extracted from caudal fin tissue using the Qiagen DNeasy 96 kit. A total of 19 microsatellite markers were used for the study: *CypG3*, *CypG4*, *CypG23*, *CypG25*, *CypG28*, *CypG35*, *CypG39*, *CypG40*, *CypG43*, *CypG45*, *CypG48*, *CypG52*, *CypG53*, *Pmac1*, *Pmac4*, *Pmac19*, *Pmac24*, *Pmac25*, *Pmac35* (Baerwald and May 2004; Mahardja et al. 2012). Polymerase chain reaction (PCR) was performed in a 20 µl reaction volume containing ~20 ng of DNA template, 0.65 units of FastStart Taq polymerase (Roche), 1X PCR reaction buffer (2 µM MgCl$_2$), 0.2 mM of each dNTP, and 0.12–0.7 µM of each primer. With the exception of *CypG28*, all markers were multiplexed in groups of 3 (Table 2). Forward primer for each locus is labeled with one of three fluorescent dyes: 6-FAM, VIC, NED. PCR thermal cycling conditions consist of initial denaturation step for 4 min at 95 °C, and 26 cycles of 30 s denaturation at 95 °C, 30 s annealing at 58 °C, 45 s extension at 72 °C, and a final extension step at 60 °C for 45 min. Capillary electrophoresis was conducted on ABI 3730 Genetic Analyzer (Life Technologies) by using 1.0 µl of each PCR product added into a mixture of 8.8 µl of highly deionized formamide and 0.2 µl of GeneScan 400HD ROX size standard (Life Technologies). Microsatellite alleles were subsequently genotyped using GeneMapper™ 4.0 (Life Technologies).

Inference of Population Structure

We used the Bayesian model-based clustering method of STRUCTURE 2.3.3 (Pritchard et al. 2000) to ensure that splittail population structure remains stable over time and to investigate any further substructure (e.g., interannual variation, spatially segregated subpopulations within each DPS). We examined the log-likelihood for $K = 1$–8 using the full dataset with 15 independent iterations per K, 250,000 burn-in period and 500,000 Markov chain Monte Carlo (MCMC) repetitions. We inferred the proper K using the estimated mean log-likelihood value (Ln Pr(X|K)) method (Pritchard et al. 2000), and when the highest likelihood value lies at $K > 1$, we also used the ad hoc ΔK method (Evanno et al. 2005) to test for concordance. To evaluate the presence of cryptic subpopulations, we conducted additional K inference tests with identical parameters using subsets of our data (2011 and 2012 San Pablo Bay collection, 2011 Central Valley collection, and the two populations post-assignment; see Fig. S2-S7).

Table 2 Characteristics of microsatellite PCR multiplexes and associated statistics assessed

	Concentration (µM)	Fluorescent label	Size range (bp)	A_R for SPB, CV
Multiplex set 1				
CypG3	0.4	NED	166–266	11.2, 14.8
CypG23	0.4	VIC	161–253	11.8, 15.7
CypG40	0.5	6-FAM	206–304	13.5, 19.3
Multiplex set 2				
CypG4	0.5	VIC	140–176	6.99, 7.89
CypG35	0.5	6-FAM	197–225	4.73, 6.33
CypG45	0.7	NED	128–132	2.00, 2.00
Multiplex set 3				
CypG25	0.2	6-FAM	122–156	6.88, 9.39
CypG39	0.3	NED	154–174	3.00, 3.81
CypG48	0.2	VIC	158–244	9.38, 12.7
Multiplex set 4				
CypG43	0.5	NED	138–276	14.3, 19.0
CypG52	0.4	VIC	112–132	4.09, 3.68
CypG53	0.3	6-FAM	150–206	10.8, 12.7
Multiplex set 5				
Pmac19	0.2	NED	135–155	3.10, 4.36
Pmac24	0.12	VIC	194–227	4.40, 6.91
Pmac25	0.45	6-FAM	212–252	7.00, 9.46
Multiplex set 6				
Pmac1	0.25	NED	146–190	9.10, 10.7
Pmac4	0.15	VIC	181–241	6.61, 8.89
Pmac35	0.3	6-FAM	226–266	7.21, 9.41
Set 7				
CypG28	0.2	NED	172–188	3.78, 5.02

Primer concentration for a single PCR reaction listed refers to the concentration of each forward and reverse primer. A_R values were rarified for 152 allele copies for the pooled samples of genetically assigned San Pablo Bay (SPB) population and Central Valley (CV) population

Results pertaining to K inference were summarized using the program STRUCTURE HARVESTER (Earl and von-Holdt 2011).

Population assignment

Extended STRUCTURE runs were used to determine the population origin of each sample (Pritchard et al. 2000) once the proper K was selected. Ten iterations of $K = 2$ were performed with all individuals included, no prior location information, 500,000 burn-in period, and 1,000,000 MCMC repetitions under the assumption of admixture and correlated allele frequencies. Replicate runs were averaged in CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) using the *FullSearch* algorithm. We defined

individuals as belonging to a specific population as those assigned with $\geq 80\%$ probability (q value ≥ 0.8) based on the efficiency and accuracy scores in the simulation study done by Vähä and Primmer (2006). Individuals whose q values fall below the threshold for both populations were considered admixed. Due to the co-occurrence of two populations in certain locations, we will hereby refer to "population" when referring to a group of samples where individuals have been assigned to a population of origin and putative migrants have been removed. Meanwhile, "collections" will refer to all samples from a location or region regardless of population assignment results.

Intra and inter-population genetic variation

Exact tests of Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were conducted using GENEPOP 4.1.1 (Raymond and Rousset 1995), with each population analyzed separately to avoid detection of the Wahlund effect. The Markov chain method was used with 10,000 dememorization steps, 100 batches, and 10,000 iterations per batch. Statistical significance was determined after sequential Bonferroni correction using a $P < 0.05$ cut-off value (Rice 1989). The presence of null alleles for each locus was examined with MICRO-CHECKER (Van Oosterhout et al. 2004). Allelic richness (A_R) values for each cohort were obtained through the rarefaction procedure to correct for differences in sample size using HP-RARE (Kalinowski 2005). Each cohort was rarified according to the smallest sample size (152 allele copies for the 2002 San Pablo Bay cohort). Observed (H_O) and expected heterozygosities (H_E) were calculated using the software ARLEQUIN v 3.5 (Excoffier et al. 2005).

Genetic differentiation level between the two populations was assessed with pairwise values of R_{ST} (Slatkin 1995) using ARLEQUIN v 3.5 (Excoffier et al. 2005) and D_{EST} (Jost 2008) using SMOGD v 1.2.5 (Crawford 2010). We also tested each population by cohort for recent bottlenecks using the M-ratio test (Garza and Williamson 2001). We used the program ARLEQUIN v 3.5 to calculate M-ratio for each cohort and M-CRIT (Garza and Williamson 2001) to determine the critical M-ratio for $P < 0.05$. Critical M-ratio numbers were acquired by using $\theta = 10$, 22% mutations greater than one step, and the average size of multiple step mutation of 3.1 based on Peery et al.'s (2012) review of microsatellite evolution studies.

Estimation of effective number of breeders (N_b) and population size (N_e)

We chose three different methods of estimating N_e based on their suitability with splittail biology and our dataset:

the unbiased linkage disequilibrium method (LDNe), the temporal F-statistic moments method, and the maximum-likelihood method (MLNe). Most N_e estimators assume discrete generations for the population of study and when species with overlapping generations are treated as discrete, a substantial amount of bias may be produced (Waples and Yokota 2007; Luikart et al. 2010). A single cohort may be sampled to estimate the effective number of breeders (N_b) during a given season to avoid violating this assumption (Waples 2005). N_b can be used as a parameter to explain the genetic changes in a cohort in place of N_e, as it is similarly affected by factors such as unequal sex ratio and the variance in contribution among parents (Wang 2009). Estimating N_b is possible when using a single-sample method of estimating effective size (i.e., LDNe). Method of estimating N_e through linkage disequilibrium was originally developed by Hill (1981) and a sample size bias correction was added by Waples (2006). This method is based on the concept that a set of unlinked loci would exhibit linkage disequilibrium due to random sampling and genetic drift. Therefore, the amount of linkage disequilibrium within the sample is expected to be inversely proportional to the size of N_e/N_b. We used the program LDNe (Waples and Do 2008) to estimate N_b in our study and to balance the precision-bias tradeoff, alleles with frequency <0.02 were removed per Waples and Do's (2010) suggestion. LDNe also assumes a closed population and due to the significant amount of migrants present in certain cohort samples (see results), migrants identified through our population assignment analysis are removed for the LDNe analysis (Waples and England 2011).

We also applied the temporal method of estimating N_e due to the sufficient time gap (>1 splittail generation) between our sampling sessions (2002–2003, 2011–2012). The temporal F-statistics method calculates a harmonic mean of N_e over two time points based on the variance of neutral allele frequencies between two temporally segregated samples (Waples 1989, 2005). Unless estimates of age-specific survival and birth rate are known, methods of calculating N_e are typically constrained to the assumption of discrete generations (Jorde and Ryman 1995). Consequently, the presence of overlapping generations will often upwardly bias F, leading to a downward bias in N_e (Waples 2005). However, as the number of generations between samples increases, the overlapping generation bias in N_e estimate will become reduced due to the larger genetic drift signal relative to noise (Waples and Yokota 2007). Three measurements of variance in allele frequencies were used for this study: F_c (Nei and Tajima 1981), F_k (Pollak 1983), and F_s (Jorde and Ryman 2007). Calculations for F', which are corrected for sample size, were conducted in the GONe software package (Coombs et al.

2012). N_e estimates were acquired using Eq. 5 in Jorde and Ryman (2007) as follows

$$N_e = \frac{t}{2F'}$$

where t is the number of generations between the two samples. Sampling plan II was selected due to the lack of census size numbers for either splittail population and 95 % confidence intervals for these estimates were calculated using Eq. 16 from Waples (1989):

$$(1 - \alpha)\text{CI for } \hat{F} = \left[\frac{n\hat{F}}{X^2_{\left(\frac{\alpha}{2}\right)[n]}}, \frac{n\hat{F}}{X^2_{\left(1-\frac{\alpha}{2}\right)[n]}} \right]$$

Estimates for the number of generations between the two temporal samples were acquired by dividing the number of years elapsed between the two sampling sessions by the average age of adult splittail (~ 4.4 years for the San Pablo Bay population, ~ 4.5 years for the Central Valley population) captured in the San Pablo Bay based on otolith and scale data (Hobbs 2013). Putative migrants were removed for this analysis as the temporal F-statistics method assumes no gene flow between populations.

The previously mentioned N_e estimators assume that each population is isolated from gene flow/immigration. But even when occurring at a low rate, migration may substantially alter the rate of change in the genetic makeup of a population, leading to under- or overestimation of N_e. To further assess the conformity of our harmonic mean N_e estimates and evaluate the effect of gene flow on N_e calculations, we used the MLNe software package (Wang 2001; Wang and Whitlock 2003). MLNe uses a pseudo-maximum-likelihood approach to provide an estimate of temporal N_e with the option of incorporating gene flow into the analysis, assuming a small focal population and an infinitely large immigration source. Harmonic N_e for each population was estimated using MLNe in two ways. First, we estimated N_e under the assumption of closed population with putative migrants excluded. Second, we conducted a joint estimation of N_e and migration rate (open population) with migrants included for comparison. Acquired migration rate estimates are not reported here due to the questionable origin and eventual fate of the Central Valley fish found in Petaluma and Napa Rivers during 2011 (see results and discussion). The Central Valley 2011 collection was designated as the immigration source when estimating the San Pablo Bay N_e under the open population assumption, while the San Pablo Bay 2012 collection was selected as the immigration source when estimating Central Valley N_e. Difference in mean N_e estimates between the two populations was evaluated by using Welch's t test. A more extensive discussion on we addressed some of the more general assumptions

associated with N_e and N_b estimators can be found in the Appendix Supplemental Methods.

Results

Inference of population structure

We genotyped 1,526 age-0 fish belonging to four year classes (2002, 2003, 2011, 2012) at 19 microsatellite loci. Number of alleles per locus ranged from 2 (*CypG45*) to 29 (*CypG43*). The level of genetic differentiation between the San Pablo Bay and Central Valley populations appears to be temporally stable. In concordance with Baerwald et al. (2007), evaluation of Ln Pr(X|K) and ΔK for the full data set (composed of samples collected in 2002–2003 and 2011–2012) indicates that the most likely number of population clusters is two (Figs. S1, S8). No further substructure was detected when STRUCTURE was run separately by collection and assigned population (putative migrants removed), as $K = 1$ resulted in the highest mean log-likelihood for all cases with the exception of San Pablo Bay 2011 collection (Figs. S2–S7).

Population assignment

STRUCTURE analysis assigned a total of 713 age-0 individuals to the San Pablo Bay population, 748 individuals to the Central Valley population, and 65 as admixed individuals out of 1,526. Average q value within the San Pablo Bay population and Central Valley population is 0.972 and 0.967 respectively, while the q-value average for the admixed group is 0.523 for the San Pablo Bay population cluster and 0.477 for the Central Valley population cluster. Results of population assignment analysis for the 2002 and 2003 cohorts remained consistent with the findings of Baerwald et al. (2007) despite the inclusion of six additional microsatellite loci. The majority of the 2002–2003 Central Valley splittail collection was assigned back into the Central Valley population (94.4 %). Similarly, a large portion of the 2002–2003 collection from the Petaluma River (96.9 %) and the Napa River (79.8 %) assigned to the San Pablo Bay population. No Central Valley fish were found within the 2002–2003 San Pablo Bay collection, with the exception of fourteen individuals collected from the Napa River in 2003 (Table 1; Fig. 2).

Population assignment of the 2011 San Pablo Bay splittail collection revealed a pattern in contrast to that of 2002–2003. Though the Central Valley region remained mainly composed of Central Valley splittail (96.1 %) in 2011, San Pablo Bay splittail formed less than half of the fish collected in the Petaluma and Napa Rivers (Fig. 3). Only 37.5 % of fish collected in the Petaluma River and
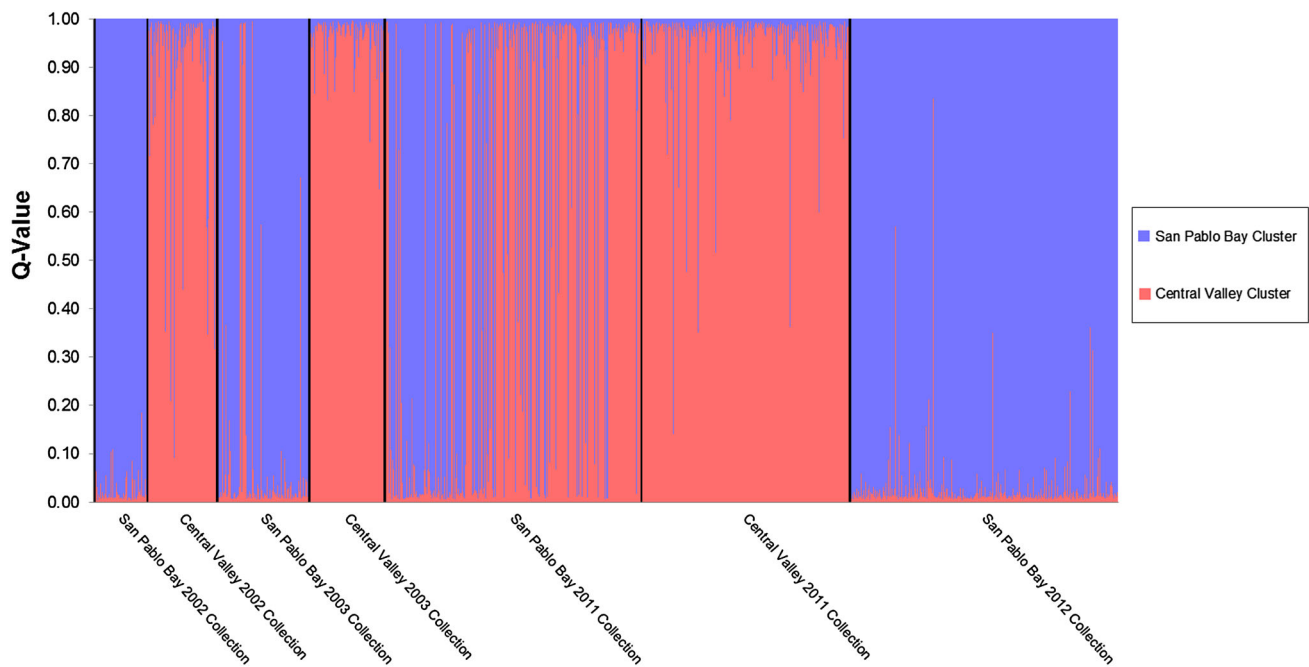
Fig. 2 STRUCTURE bar histogram depicting individual *q* value assignments for the study's full data set. Three samples collected from China Camp State Park were included into the San Pablo Bay 2011 collection

24.7 % in the Napa River assigned back into the San Pablo Bay population in 2011, with 55.3 % in the Petaluma River and 73.0 % in the Napa River assigning to the Central Valley population. Moreover, three splittail collected in China Camp State Park, the closest sampling point to the mouth of San Francisco Bay, all assigned into the Central Valley population. However, this pattern was not observed in the 2012 San Pablo Bay collection. Of the 403 age-0 splittail collected from Napa River in 2012, 96.3 % assigned to the San Pablo Bay population and <1 % (2 individuals) assigned to the Central Valley population.

Intra and inter-population genetic variation

Average A_R across loci for the assigned San Pablo Bay population and Central Valley population yearly cohorts ranged from 7.20 to 7.26 and 9.43 to 9.55, respectively. Significant departures from HWE after Bonferonni adjustment were observed in *CypG53* for the collective San Pablo Bay population (all cohorts with putative migrants removed), and in *CypG3*, *Pmac25* for the Central Valley population. HWE deviations for *CypG53* and *Pmac25* appear to be due to the presence of null alleles for their respective populations based on MICRO-CHECKER analysis. Of the 171 possible pairs of loci, four pairs (*CypG3*/*CypG40*, *CypG3*/*Pmac25*, *CypG25*/*CypG48*, *CypG39*/*CypG52*) in the San Pablo Bay population, and one pair (*CypG40*/*Pmac25*) in the Central Valley population

exhibited LD after sequential Bonferroni correction. Due to the lack of congruence in LD between the STRUCTURE assigned populations, the presence of true physical linkage between our microsatellite markers seems unlikely.

Pairwise R_{ST} comparisons between assigned cohorts found significant differences only when comparisons were made between cohorts of different populations, and not within (Table 3). When comparing the two populations, significant genetic divergence was detected at an R_{ST} value of 0.036. Jost's measure of differentiation, D_{EST}, showed a similar level of differentiation between the two populations with a harmonic mean of 0.069 across loci. Post-assignment, M-ratio bottleneck test was significant for the 2012 San Pablo Bay cohort, while the values for the remaining assigned cohorts from either population were all above their respective critical values. Although results from this test should be interpreted with caution because the proportion of multistep mutations is often underestimated (Peery et al. 2012), averaged M-ratio values across loci for the assigned Central Valley cohorts were also consistently higher than those of San Pablo Bay cohorts. The M-ratio disparity between the two populations reflects the higher genetic diversity for the Central Valley population, as predicted by their expected larger census population size. M-ratio values for each assigned Central Valley cohort were 0.845, 0.846, and 0.910 for 2002, 2003, and 2011, respectively. Meanwhile, M-ratio values for the San Pablo Bay cohorts were 0.747, 0.766, 0.778, and 0.774 for 2002, 2003, 2011, and 2012.
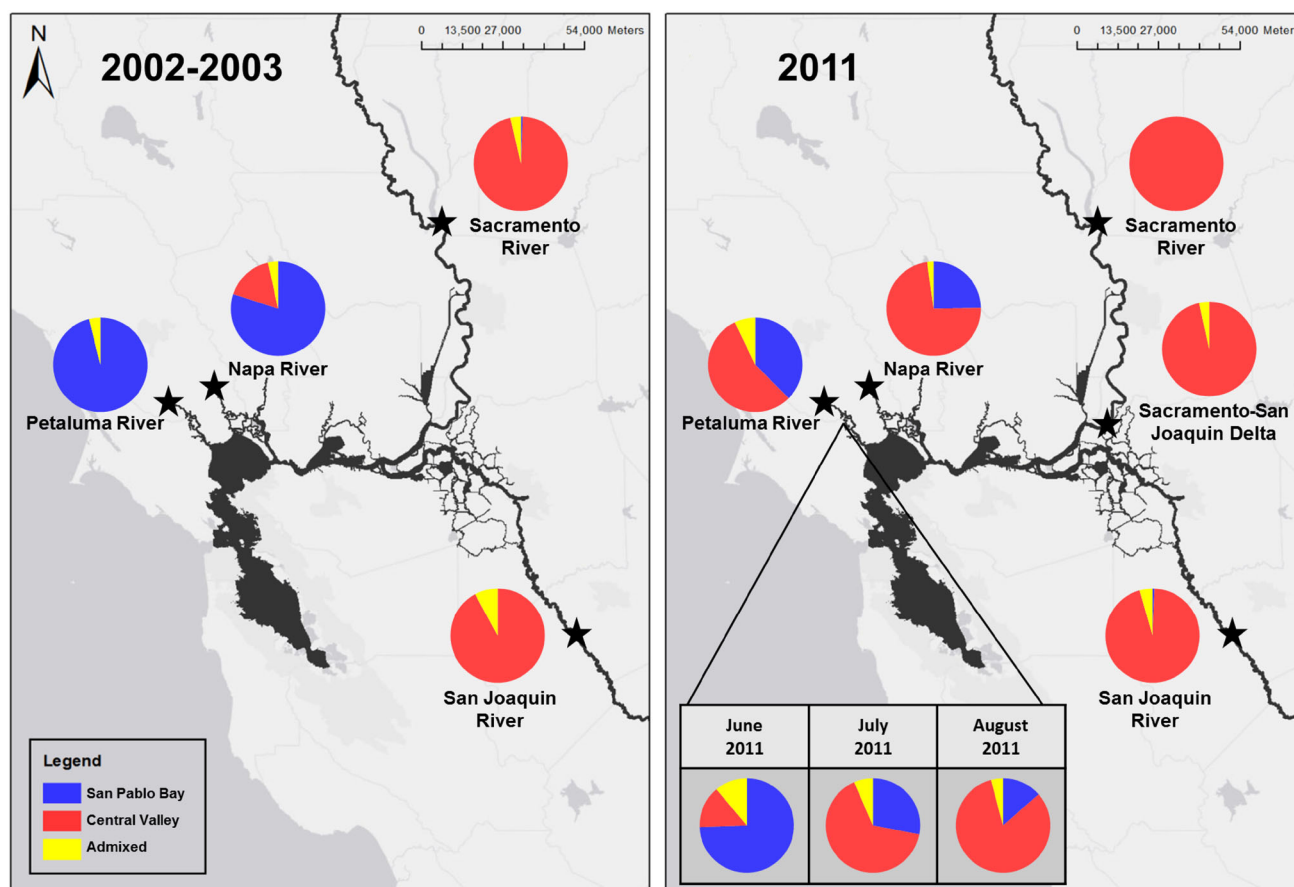
Fig. 3 Map overview of sampling regions with their respective population assignment results proportions. Although the two splittail populations were relatively spatially segregated in 2002–2003, extensive overlap in range was observed in the high rainfall year of 2011. A pattern of increasing Central Valley population dominance within the Petaluma River was observed in 2011 when samples are partitioned into the three sampling months ($N = 90$ for June, $N = 107$ for July, and $N = 96$ for August), suggesting the possibility of movement into this tributary

Table 3 Pairwise R_{ST} (Slatkin 1995) (below the diagonal) and harmonic mean of D_{EST} (Jost 2008) values across loci (above the diagonal) for the various age-0 splittail cohorts of the two populations with putative migrants removed

	San Pablo Bay 2002	San Pablo Bay 2003	San Pablo Bay 2011	San Pablo Bay 2012	Central Valley 2002	Central Valley 2003	Central Valley 2011
San Pablo Bay 2002	–	0.000	0.001	0.000	0.066	0.081	0.076
San Pablo Bay 2003	0.000	–	0.002	0.002	0.065	0.074	0.073
San Pablo Bay 2011	0.000	0.000	–	0.002	0.053	0.067	0.061
San Pablo Bay 2012	0.000	0.002	0.001	–	0.064	0.074	0.068
Central Valley 2002	0.024*	0.027*	0.029*	0.033*	–	0.001	0.000
Central Valley 2003	0.029*	0.036*	0.032*	0.037*	0.002	–	0.000
Central Valley 2011	0.034*	0.040*	0.037*	0.042*	0.000	0.000	–

* Significant differentiation for R_{ST} comparison ($P < 0.05$)

Estimates of N_b and N_e

Our estimates of contemporary N_b for the San Pablo Bay population were generally lower than the Central Valley population (Table 4). Excluding the 2002 San Pablo Bay cohort due to its combination of smaller sample size (n = 76) and imprecise estimate (infinite upper confidence limit), the mean estimates of N_b for the San Pablo Bay population based on LDNe ranged from 317 to 447, while the mean estimates for the Central Valley population ranged from 540 to 14,393. The temporal F-statistics methods yielded a N_e average across pairwise cohort comparisons of

Table 4 Estimates of effective number of breeders (N_b) with their respective 95 % confidence interval in parentheses based on LDNe, expected heterozygosity (H_E), observed heterozygosity (H_O), and allelic richness (A_R) values for 152 allele copies for the various age-0 cohorts of the two splittail populations

Population	Year	Sample size	H_E	H_O	A_R	N_b
Central Valley	2002	102	0.64	0.62	9.47	953 (470–37,386)
	2003	111	0.63	0.63	9.43	540 (343–1185)
	2011	311	0.63	0.62	9.55	14,393 (2345–∞)
San Pablo Bay	2002	76	0.65	0.65	7.63	1,843 (418.4–∞)
	2003	122	0.66	0.64	7.26	317 (230–491)
	2011	131	0.65	0.64	7.41	334 (253–478)
	2012	402	0.65	0.64	7.2	447 (378–541)

Putative migrants were removed for this analysis due to potential introduced bias

424 for the San Pablo Bay population and 1,037 for the Central Valley population (Table 5). Estimates obtained using the pseudo-maximum-likelihood approach also gave similar results when populations were assumed to be closed. San Pablo Bay population yielded N_e estimates ranging from 219 to 485, while estimates for the Central Valley population were 1,641 and 4,903 with infinite upper limits. Reduction in N_e estimates for both populations was observed when migration was considered, though it appears to be more substantial for the San Pablo Bay population. Overall, mean N_e estimates acquired from the various temporal methods was also significantly lower for the San Pablo Bay population (Welch's t test = 2.8, df = 9.5, P = 0.020).

Discussion

Population Structure

We observed two genetic clusters among age-0 splittail collected in 2011–2012 as was previously found in 2002–2003 (Baerwald et al. 2007), but in contrast to Baerwald et al.'s (2007) study, there was an extensive geographical overlap between the two genetically distinct populations in 2011 (Fig. 3). Although the Petaluma and Napa Rivers contained mainly individuals from the San Pablo Bay population in most years (2002, 2003, 2012), Central Valley individuals formed a majority (59 %) of the age-0 fish collected in this region during the 2011 sampling period. One possible explanation is that the presence of age-0 Central Valley splittail within these rivers resulted from an increased spawning by Central Valley adult splittail in the San Pablo Bay region. However, there are indications that these age-0 Central Valley splittail originated further upstream instead and moved into the Petaluma and Napa Rivers early in their juvenile life stage.

Splittail possess an unusually high salinity tolerance among cyprinids, which typically consist of stenohaline freshwater fishes. This salinity tolerance generally increases with size and age, where some adult splittail can endure salinities temporarily up to 29 ppt (parts per thousand) under a laboratory setting (Young and Cech 1996). Meanwhile, age-0 splittail cannot endure high salinity levels to the same extent as their adult counterparts and experience loss of equilibrium at salinities >22 ppt under the same laboratory condition (Young and Cech 1996). As such, it has been postulated that salinity is one of the key limiting factors in the distribution of juvenile splittail in the San Francisco Estuary. Feyrer et al. (2010) proposed that during an average or dry year, highly saline water found in San Pablo Bay and Carquinez Strait would effectively act as a barrier between the two splittail populations. During a wet year however, high freshwater outflow from the Sacramento River and San Joaquin River may reduce the salinity level within the upper San Francisco Estuary, temporarily removing this salinity barrier. This breach in the salinity barrier, coupled with the presumed increased abundance of age-0 Central Valley splittail from the expansion of freshwater spawning habitat could allow

Table 5 Estimates of harmonic mean effective population size (N_e) of the two splittail populations by various temporal methods and cohort comparisons and their respective 95 % confidence intervals in parentheses

Year comparison	Moments, Fc (Nei and Tajima 1981)	Moments, Fk (Pollak 1983)	Moments, Fs (Jorde and Ryman 2007)	ML, closed (Wang and Whitlock 2003)–migrants excluded	ML, open (Wang and Whitlock 2003)–migrants included
San Pablo Bay 2002–2011	394 (314–484)	386 (307–474)	252 (200–309)	471 (222–6,074)	196 (123–363)
San Pablo Bay 2002–2012	617 (492–756)	542 (432–663)	1,357 (1,082–1,662)	485 (279–1,285)	74 (71–81)
San Pablo Bay 2003–2011	181 (143–222)	183 (145–225)	125 (100–154)	219 (132–532)	194 (158–246)
San Pablo Bay 2003–2012	392 (313–479)	406 (325–497)	256 (204–313)	472 (294–967)	63 (60–66)
Central Valley 2002–2011	1,015 (839–1,207)	976 (807–1,161)	640 (529–762)	1,641 (632–∞)	1,202 (525–40,811)
Central Valley 2003–2011	1,320 (1,092–1,570)	1,287 (1,064–1,530)	862 (713–1,025)	4,903 (601–∞)	877 (344–∞)

N_e estimates were generally lower for the San Pablo Bay population, with mean and median of 363.25 and 321 respectively, while mean and median of the Central Valley population were 1,472 and 1,109

Fig. 4 Net water outflow from the Sacramento-San Joaquin Delta for the water years that the samples were collected. California water year begins on October 1st and ends on September 30th, and is designated by the calendar year which it ends (e.g. water year 2011 begins in October 2010 and ends in September 2011). The years of 2002 and 2003 were more comparable to the decade average (**a**) than the relatively wet and dry year of 2011 and 2012 (**b**). Also demonstrated is the much higher outflow during the exceptionally wet year of 2006 (**c**) even in comparison to 2011
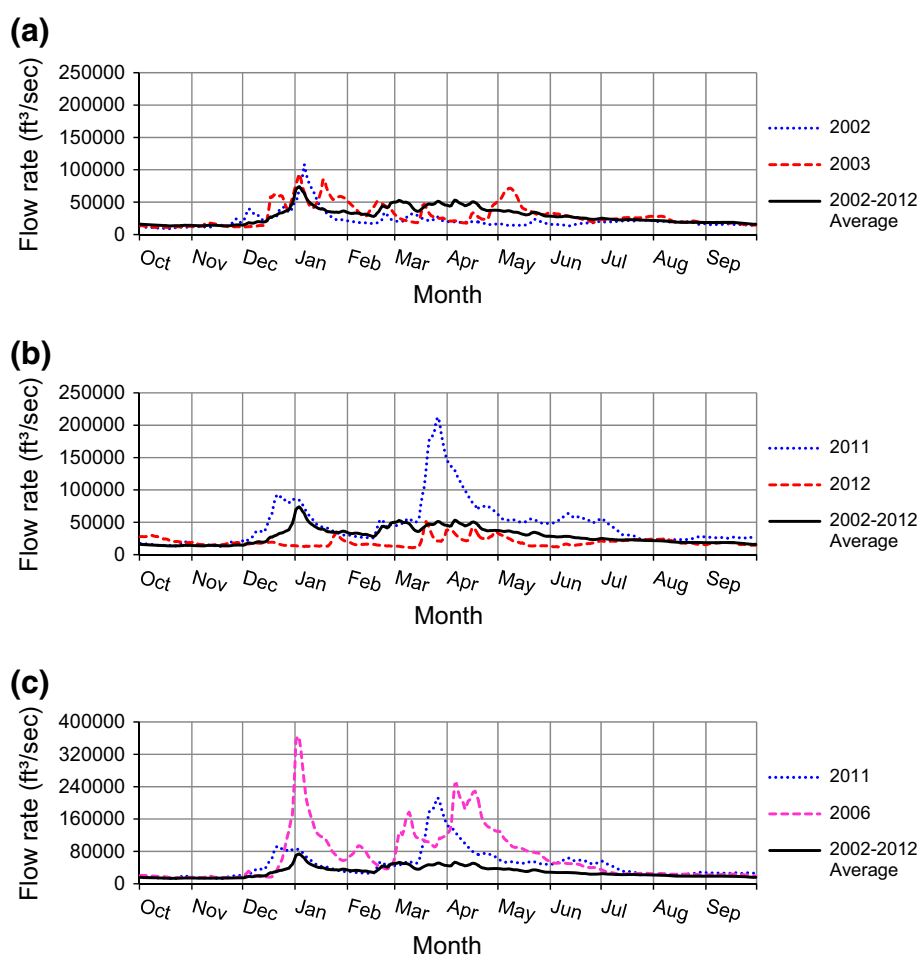
movement of Central Valley origin age-0 splittail into the Petaluma and Napa Rivers.

The Net Delta Outflow Index (http://www.water.ca.gov/dayflow/output/), a proxy for water volume exiting the Sacramento-San Joaquin Delta into San Francisco Bay calculated by the California Department of Water Resources DAYFLOW model, provided corroborating evidence for this hypothesis (Figs. 4a, b, 5a, b). The years of 2002 and 2003 generally followed the average pattern of the Delta freshwater outflow for the past decade (2002–2012) (Fig. 5a). On the other hand, 2011 was considered a wetter year than average, with an elevated Delta outflow between late March and early May (Fig. 4b). This spring flow pulse would have rendered the eastern portion of San Pablo Bay sufficiently fresh for juvenile splittail to settle into. The reduction in salinity within the San Francisco Estuary can also be seen through the DAYFLOW X2 calculations (the distance in kilometers from San Francisco Golden Gate Bridge along the thalweg to the near-bed water with salinity of 2 ppt) during this period (Fig. 5a, b).

The hypothesis for age-0 splittail movement is also supported by the temporal pattern in our population

assignment analysis results. When 2011 Petaluma and Napa River samples were partitioned by month, we observed an increasingly higher Central Valley influence over time (Fig. 3). The proportion of Central Valley fish in both Petaluma and Napa Rivers in 2011 increased from 19 % in June, to 66 % in July, and 86 % in August. At the same time that this population shift took place, the position of the X2 isohaline was steadily moving upstream (Fig. 5a, b). In effect, higher salinity water lethal to juvenile Central Valley splittail was returning to San Pablo Bay and Carquinez Strait during this time period. The encroachment of high salinity water suggests that the increase in age-0 Central Valley splittail within the Petaluma and Napa Rivers may be a result of a movement upstream in order to avoid the highly saline water. Field records are in agreement with this interpretation, as salinity measurements and number of splittail catches per seine were higher in the months of July and August (unpublished data).

STRUCTURE analysis clearly differentiated Central Valley splittail from San Pablo Bay splittail. Had the Petaluma and Napa Rivers been the true natal origin of the Central Valley splittail in the San Pablo Bay 2011

Fig. 5 X2 measurement (the distance in kilometers from Golden Gate Bridge along the thalweg to the near-bed water with salinity of 2 ppt) for the San Francisco Bay for the various the water years that samples were collected in X2 is highly correlated with the net outflow from the Sacramento-San Joaquin Delta, where it is closer to the opening of San Francisco Bay to the Pacific Ocean during wet years and farther during dry years. The year 2002 and 2003 were average years with little deviation from the decade average (**a**), while 2011 and 2012 were considered above and below average years in precipitation (**b**). The year 2006 and 2011 were wet years (**c**) in which the salinity barrier to splittail migration was expected to be lacking
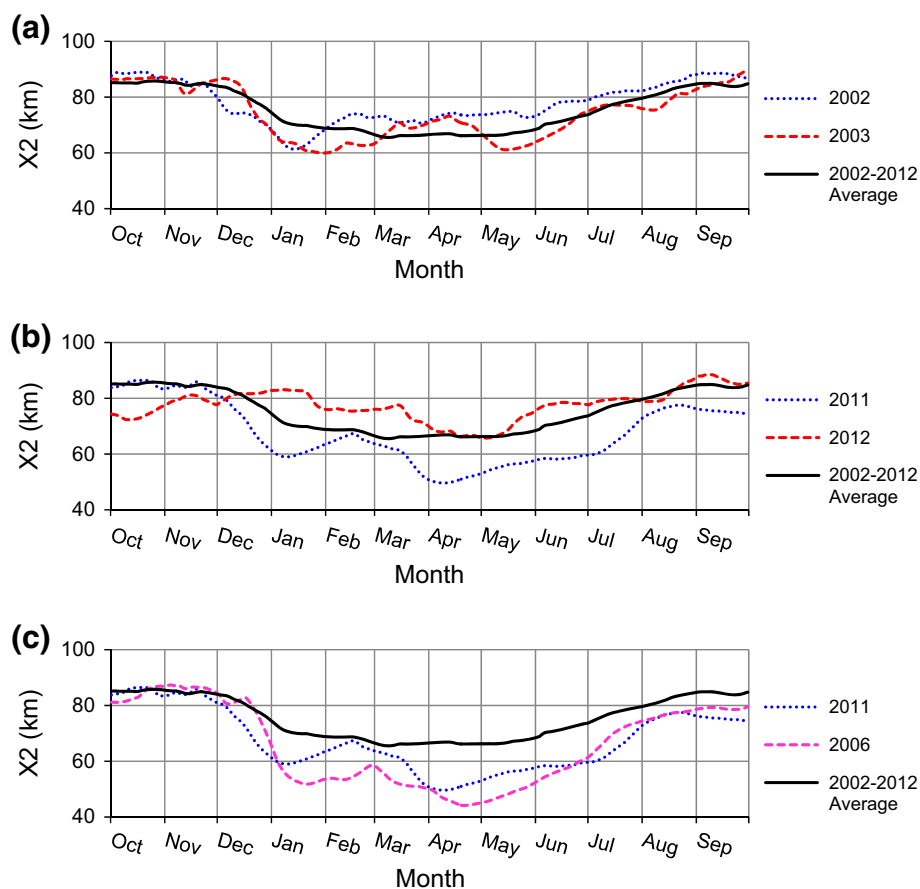
collection, strong selective mating and/or outbreeding depression would have been required to preserve this genetic differentiation. Nonetheless, this alternate hypothesis cannot be dismissed without confirmation from otolith microchemistry data.

To examine if the increased Central Valley splittail presence is observed again the following year, we collected age-0 fish in the Petaluma and Napa Rivers in 2012. Unlike 2011, 2012 had below average precipitation, as reflected in the Delta outflow and X2 measurements (Figs. 4b, 5b). Attempts to collect age-0 splittail in Petaluma River failed during 2012, and we encountered water quality conditions at the supposed salinity tolerance limit range for juvenile splittail based on Young and Cech's (1996) study within this location (average of 21 ppt and 29 °C). This unfavorable water condition may have either prevented or significantly reduced splittail recruitment in the Petaluma River in 2012. Field collection from the Napa River in 2012 was successful however, with a total of 403 fish captured. Population assignment of 2012 Napa River collection indicated that the region has reverted to a San Pablo Bay population majority. Of the 403 age-0 splittail sampled within the Napa River in 2012, 388 assigned to the San Pablo Bay population, 2 assigned to the Central Valley

population, and the remaining samples were classified as admixed.

Estimates of N_b and N_e

Given the larger region of apparent spawning habitat within the Central Valley and higher genetic diversity (A_R, M-ratio), annual N_b is expected be larger for the Central Valley splittail population in comparison to the San Pablo Bay population. This is in agreement with the linkage disequilibrium N_b estimates. With the exception of the 2002 San Pablo Bay cohort with its limited sample size, the mean N_b estimates for the Central Valley population were consistently higher than that for the San Pablo Bay population (Table 4). Furthermore, there was greater uncertainty and year-to-year variability in the N_b estimates (reflected by the infinite CIs) in the Central Valley cohorts despite comparable sample size to the San Pablo Bay cohorts. This may be a reflection of Central Valley population's larger true N_e, as the LDNe method has greater power when true effective size is small (Waples 2006).

Similar to N_b estimates, temporal N_e estimates were generally higher and more imprecise for the Central Valley splittail population than the San Pablo Bay population.

Although this low precision makes the comparison between the Central Valley population's N_b and N_e difficult, San Pablo Bay population N_e and N_b estimates were relatively precise and fell within similar range (~ 300–400). This N_b/N_e ratio of roughly one is fairly typical among iteroparous fish species (Serbezov et al. 2012; Duong et al. 2013; Waples et al. 2013), and appears to be consistent with Waples et al.'s (2013) finding that link a species' age of maturation and adult lifespan with its N_b/N_e ratio.

Conservation implications

Our study demonstrates that the interaction between the two genetically distinct populations of splittail is more complex than previously thought. Baerwald et al. (2007) proposed that this genetic differentiation were a result of separate foraging and rearing habitats, as well as a high salinity barrier between the two populations. However, overlap in foraging distribution between the two populations was subsequently found (Baerwald et al. 2008) and our results demonstrated extensive connectivity among age-0 fish during wet years. Due to the listing of these two populations as distinct population segments (USFWS 2010), it is crucial to assess the possibility of increased gene flow in the near future.

If the observed increased geographical overlap in this study is a direct result of the high freshwater outflow in 2011, we can then assume that a similar occurrence also took place in 2006, when precipitation was higher than 2011 (Figs. 4c, 5c). Under this assumption, the increased number of 2006 age-0 Central Valley splittail in Petaluma and Napa Rivers would eventually mature and distort the genetic identity of the two populations. Nevertheless, we continued to observe distinct genetic clusters in 2011 and 2012, and found only a small proportion of unassigned/admixed individuals. This suggests that one or more mechanisms continue to reproductively isolate the two populations. Some plausible explanations include: disruptive selection (a type of natural selection that selects for opposite extremes of a trait and selects against the average/middle) based on differences in physiological traits (e.g. salinity tolerance), and/or homing behavior exhibited by the species similar to that in salmonids. Regardless of the mechanism however, the absence of apparent increased admixture in 2011 and 2012 after the wet year in 2006 suggests that migrants from the Central Valley do not augment the San Pablo Bay population to a considerable amount.

Our effective population size estimations also confirmed the common assumption that the Central Valley splittail population is more robust than the San Pablo Bay population. It has been suggested that a minimum N_e of 50 is required to avoid negative effects of inbreeding, while N_e of ~ 500–1000 is necessary to maintain evolutionary potential (Franklin 1980; Franklin and Frankham 1998; Lynch and Lande 1998). Our N_e and N_b estimations for the Central Valley population generally meet these thresholds, suggesting that negative impact due to genetic factors is unlikely for this population. Yet N_e and N_b estimations for the San Pablo Bay splittail population often fall below 500, though they always remain above the critical threshold of 50. Although the relationship between adaptive quantitative traits and neutral genetic diversity may be unclear at times (Willi et al. 2006), these relatively low effective size numbers is a concern when considering the possible effects of climate change.

Future projections of the San Francisco Estuary predict increasing water temperatures, salinity and sea level accompanied by decreasing precipitation and runoff (Cloern et al. 2011). Additionally, the morphology of the San Pablo Bay region has been significantly altered from past diking of natural tidal marshes, reducing available shallow edge habitats essential to splittail (Moyle et al. 2004). While splittail can be considered a eurythermal and euryhaline species (Young and Cech 1996), and it has been hypothesized that San Pablo Bay splittail are less reliant on freshwater overall (Baerwald et al. 2007; Feyrer et al. 2010), successful spawning for this species generally require a relatively narrow combination of environmental factors (Moyle et al. 2004). Due to the possibility of Petaluma and Napa Rivers becoming increasingly unsuitable for spawning and rearing of splittail in the future, we recommend that an abundance and/or genetic effective population size monitoring of the San Pablo Bay splittail population be conducted at least once per generation (~ 4 years).

Conclusions

Our study discovered an extensive overlap in the range of age-0 splittail from two genetically distinct populations (Central Valley and San Pablo Bay populations) within the Petaluma and Napa Rivers. This range overlap appears to be heavily influenced by high precipitation in 2011; however, we retained the ability to clearly distinguish the two genetic clusters in 2011 and 2012 after a similarly high precipitation year in 2006. This suggests that migration of Central Valley splittail into the range of the San Pablo Bay population does not typically result in a significant increase of gene flow into the San Pablo Bay population. Results from this study also demonstrated lower N_b and N_e estimates for the San Pablo Bay population, as expected based on presumed habitat availability and genetic diversity indices from previous studies. The relatively low effective size estimates for San Pablo Bay splittail, combined with the threats of climate change, highlight the need of a more

consistent monitoring effort for this less studied population.

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